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Phenanthrene-Degrading Phenotype of *Alcaligenes faecalis* AFK2

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A phenanthrene-degrading bacterium that assimilated a wide range of organic compounds was isolated from a soil sample and identified as *Alcaligenes faecalis* strain AFK2. The strain degraded phenanthrene through protocatechuate, but did not utilize naphthalene. The phenanthrene-degrading phenotype (Phn^+) of AFK2 disappeared after 20 successive subcultures in a mineral salts medium containing *o*-phthalate or after subculture in nutrient broth containing mitomycin C. The results suggested that the Phn^+ phenotype of this strain might be encoded by extrachromosomal genes.

Pseudomonas species are known to be versatile scavengers that decompose a wide range of organic compounds that other bacteria cannot utilize. Aromatic hydrocarbons, including toluene (20), naphthalene (2, 3), and phenanthrene (4, 10), have been metabolically or genetically investigated with regard to their degradative pathways almost exclusively in pseudomonads. We have studied the degradation of phenanthrene by bacteria other than *Pseudomonas* species and described a novel pathway of degradation in *Aeromonas* (12), *Vibrio* (11), and *Pseudomonas* (11) species.

We are interested in the mineralization of polycyclic aromatic hydrocarbons by bacterial populations in nature. To elucidate the mechanisms, a wide range of bacteria should be surveyed with respect to their degradative pathways. Since we have devised a technique for detecting polyaromatic hydrocarbon-degrading bacteria on solid media (13), such studies are now possible. This paper describes the isolation and identification of a phenanthrene-degrading (Phn^+) bacterial strain and derivation of Phn^- mutants from it.

MATERIALS AND METHODS

Isolation of a Phn^+ bacterium. Phn^+ strains were isolated after seven subcultures of a portion of a soil suspension in a test tube containing 10 ml of phenanthrene-MM2 medium (13), followed by cloning colonies on LB plates (13). Thirty Phn^+ strains were isolated from 100 soil samples. Among the bacterial isolates, a strain (AFK2) which was able to grow well in phenanthrene-MM2 medium and did not produce acid from D-glucose was selected for further study.

Characterization of strain AFK2. After the bacterium was cultured on an LB agar plate for 1 to 2 days, flagella were stained for microscopic examination. Cells were also observed by electron microscopy after chromium shadowing and negative staining. Physiological and biochemical properties were examined according to the procedures described by Cowan and Steel (1), Skerman (18), and Pleczar (17). Assimilation of organic compounds, chemolithotrophic growth with H_2 gas, arginine dihydrolase test, denitrification, and cleavage of an aromatic ring were examined by the methods of Stanier et al. (19). Citrate utilization was tested on Koser medium, Christensen medium, and Stanier assimilation test medium. Nitrogen fixation was judged by the method of Knowles et al. (14) based on the ability to reduce acetylene after 5 weeks of cultivation in a Pankhurst tube. DNA was extracted as described by Marmur (15), and the base composition of DNA (guanine-plus-cytosine [G+C] content) was calculated from its melting temperature.

Measurement of ability to oxidize organic compounds. AFK2 cells were cultured overnight at 30°C in a 500-ml shaking flask containing phenanthrene suspended in 100 ml of MM2 or LB medium on a reciprocal shaker. After they were harvested by centrifuging at 12,000 rpm and washed with 10 mM Na_2HPO_4-KH_2PO_4 buffer, pH 7.0 (P-buffer), they were suspended in the same buffer to a cell concentration of about 5 mg (dry weight) per ml.

Abilities of the cells to oxidize substances were measured by a Clark electrode (YSI model 53). After 0.5 ml of the cell suspension was resuspended to 2.5 ml of P-buffer stirring at 30°C in a cell, 0.1 ml of the substrate solution (10 μmol) to be tested for oxidation was injected into the cell culture through a syringe with a needle. For substrate solutions, water-soluble compounds were previously dissolved in P-buffer, and insoluble ones were dissolved in ethanol. Oxygen consumption was immediately monitored. The ability to oxidize was expressed as nanomoles of oxygen consumed per minutes per milligram of dry cells. The values were corrected for endogeneous respiration.

Curing of Phn^+ phenotype of AFK2. For curing with growth substrates, AFK2 was successively subcultured overnight at 30°C in 10 ml of MM2 containing 10

mM disodium succinate, protocatechuate, or disodium *o*-phthalate or in LB medium on a rotary shaker. A portion (0.1 ml) of each subculture was withdrawn, diluted 1:10 with P-buffer, and spread onto LB agar plates. After the plates were incubated overnight at 30°C, they were sprayed with an ethereal solution of phenanthrene (about 10%, wt/vol) and incubated overnight. Phn^+ colonies formed clear zones in the plates, and Phn^- ones formed white colonies which remained covered with the solid hydrocarbon. Curing frequency was expressed as the ratio of Phn^- clones to total colonies.

For curing with mutagens, the bacterium was cultured for 24 to 48 h in 5 ml of LB medium containing various concentrations of mitomycin C, acridine orange, ethidium bromide, or sodium dodecyl sulfate, followed by the detection of Phn^- clones by the procedure described above.

Chemicals. *cis*-3,4-Dihydro-3,4-dihydroxyphenanthrene (phenanthrene dihydrodiol) was isolated from the culture broth of *Beijerinckia* B/836 (7) in a phenanthrene-containing succinate-mineral salts medium by the procedure described by Jerina et al. (10). All other chemicals were obtained commercially.

RESULTS

Identification and properties of AFK2. Strain AFK2 was a gram-negative, nonsporulating, motile bacillus with one to eight flagella. The cells measured 0.5 to 0.8 by 1.5 to 2.5 μm. In nutrient broth, the optimal growth temperature was 25 to 37°C; it grew at 40°C but not at 45°C. At pH 6.0 to 8.0, it grew well; it grew at a pH as high as 10.0, but not at pH 4.0. No diffusible pigment was produced on *Pseudomonas* P and F media (Difco Laboratories). This strain produced oxidase, catalase, and urease, but did not show enzyme activity such as hydrolysis of starch, casein, arginine, Tween 20, esculin, gelatin, DNA, *p*-nitrophenylphosphate, or milk except for hippurate. Neither gas nor acid was produced from carbohydrates. Alkali was produced on Hugh-Leifson medium, and citrate was utilized. The G+C content of DNA was 68.4 to 68.8 mol%. The following tests were all negative: fixation of N_2, utilization of CO_2, Voges-Proskauer and methyl red tests, production of H_2S, and formation of arginine dihydrolase.

As Table 1 shows, 150 organic compounds were tested for assimilation by this strain. Of these, 72 were utilized as carbon and energy sources. In liquid medium, the strain grew well using phenanthrene, and an orange pigment was produced in the culture broth after 6 h of cultivation. Anthracene was utilized in solid but not liquid media.

According to *Bergey's Manual* (9), a peritrichously flagellated bacterium which produces oxidase and catalase and does not attack D-glucose either oxidatively or fermentatively belongs to the genus *Alcaligenes*. This genus includes four species: *A. faecalis*, *A. aquamarinus*, *A. eutrophus*, and *A. paradoxus*. Strain AFK2 was assigned to *A. faecalis* on the basis of the morphological and biochemical observations. The G+C content (68.4 to 68.8 mol%) of DNA in AFK2 showed a higher value than that (58.9 mol%) of DNA in *A. faecalis* described in *Bergey's Manual*. This value is, however, within the range of G+C contents (57.9 to 70.0 mol%) found in this genus.

Degradation of phenanthrene by *A. faecalis* AFK2. Since *Aeromonas* S45P1 as well as other Phn^+ isolates had been found to degrade phenanthrene through 1-hydroxy-2-naphthoate, 2-carboxybenzaldehyde, and *o*-phthalate (in that order) to protocatechuate (11, 12), strain AFK2 was examined for the ability to oxidize this hydrocarbon and its possible intermediates, including phenanthrene dihydrodiol (10). Cells grown in phenanthrene-MM2 and phenanthrene-LB media oxidized all of these compounds (Table 2). The results suggest that, like *Aeromonas* S45P1, this strain degrades the hydrocarbon through protocatechuate, and the pathway is possibly inducible because the cells grown in LB medium without phenanthrene did not oxidize these compounds.

Disappearance of Phn^+ phenotype of AFK2. We had previously encountered the disappearance of the Phn^+ phenotype of S45P1 (11). Therefore, to examine whether Phn^- mutants arise from the wild-type AFK2, we attempted to detect colonies which had lost the ability to form clear zones on phenanthrene-sprayed LB plates. Subcultures on LB or succinate- or protocatechuate-MM2 liquid medium gave no Phn^- strains (Table 3). However, when the strain was successively transferred in *o*-phthalate–MM2 medium, Phn^- mutants occurred at a frequency of 2.6% after subculture. The frequency rose by increasing the number of subcultures in the medium. In addition, such mutants also occurred at a frequency of 1 to 2% after growing in the presence of a mutagen such as mitomycin C, acridine orange, or sodium dodecyl sulfate. None of the strains which had not formed clear zones on phenanthrene-sprayed LB plates grew on phenanthrene-sprayed MM2 or in phenanthrene-suspended MM2 liquid medium, nor did they revert to the Phn^+ phenotype after subculture in phenanthrene LB medium.

DISCUSSION

It has been known that the soil-inhabiting bacteria *Pseudomonas* species are versatile in assimilating organic compounds, including unnatural ones. In fact, Stanier et al. (19) found that these bacteria are capable of utilizing 104 species of 145 compounds tested. On the other hand, an enteric bacterium, *Salmonella typhi-*

TABLE 1. Assimilation of organic compounds by *A. faecalis* AFK2

Type of compound	Assimilation	
	Yes	No
Sugars	Gluconate, saccharate, D-mannose, D-galactose, D-xylose, 2-ketogluconate	D-Ribose, D- and L-arabinose, D-fructose, sucrose, trehalose, maltose, cellobiose, lactose, starch, inulin, D-glucose
Fatty acids	Acetate, propionate, butyrate, isobutylate, valerate, isovalerate, *n*-hexanoate, *n*-heptanoate, *n*-octanoate, *n*-nonanoate, *n*-decanoate	
Dicarboxylic acids	Succinate, fumarate, glutarate, adipate, pimelate, suberate, azerate, sebacate, maleate	Oxalate, malonate
Hydroxy acids	DL-α-Hydroxybutyrate, DL-lactate, D- and L-malate, L-(+)-tartrate, *meso*-tartrate	Glycolate, D-(−)-tartrate
Miscellaneous acids	Citrate, pyruvate, itaconate, mesaconate	Aconitate, levulinate, citraconate
Alcohols	Ethanol, *n*-butanol, *iso*-butanol, glycerol	Methanol, *n*-propanol, isopropanol, geraniol, erythritol, mannitol, sorbitol, *meso*-inositol, adonitol, ethyleneglycol, propyleneglycol, 2,3-butyleneglycol
Amino acids	D- and L-alanines, L-glutamate, γ-aminobutyrate, L-serine, L-leucine, L-valine, L-aspargine, β-alanine, δ-aminovalerate, DL-ornithine, L-proline, L-tyrosine, DL-phenylalanine, L-tryptophan, anthranilate, L-histidine	Glycine, L-lysine, DL-norleucine, L-threonine, L-isoleucine, DL-arginine, DL-α-aminovalerate, D-tryptophan, L-kynurenate, *m*-aminobenzoate, *p*-aminobenzoate
Amines	Tryptamine, *n*-amylamine	Methylamine, ethanolamine, benzylamine, putrescine, spermine, histamine, butylamine
Miscellaneous nitrogen compounds	Hippurate, panthothenate, acetamide, nicotinate	Betaine, creatine, trigonelline, sarcosine
n-Alkanes	*n*-Dodecane	Methane, ethane, propane, butane, hexane
Aromatic hydrocarbons	Phenanthrene, anthracene	Benzene, toluene, xylenes, naphthalene, benz[α]anthracene
Aromatic acids	2-Carboxybenzoate, *m*-hydroxybenzoate, benzoate, *o*-phthalate, protocatechuate, phenylacetate	Salicylate, *o*- and *p*-hydroxybenzoate, *p*- and *m*-toluate, *iso*- and *tert*-phthalate, D- and L-mandelate, quinate, 1-hydroxy-2-naphthoate
Phenols and others		Phenol, catechol, cresol, testosterone

TABLE 2. Oxidation of possible metabolic intermediates by cells of *A. faecalis* AFK2 which were grown in phenanthrene-mineral salts medium and LB medium

Growth medium	Oxidation (nmol of O_2/min per mg of dry cells)					
	Phenanthrene	Phenanthrene dihydrodiol	1-Hydroxy-2-naphthoate	2-Carboxybenzaldehyde	*o*-Phthalate	Protocatechuate
Phenanthrene-MM2 medium	151	129	9	54	135	85
LB medium	0	NT[a]	0	0	0	0
Phenanthrene plus LB medium	56	NT	22	27	52	3

[a] NT, Not tested.

TABLE 3. Disappearance of the Phn$^+$ phenotype of *A. faecalis* AFK2 after subculture

Subculture medium	No. of subcultures	Phn$^-$ clones/ total colonies	Frequency of curing
LB	16	0/13,522	0
Succinate-MM2	15	0/459	0
Protocatechuate-MM2	15	0/1,792	0
o-Phthalate–MM2	1	0/640	0
	6	74/2,896	2.6
	11	77/752	10.2
	13	220/1,256	17.5
	14	620/1,778	34.9
	20	742/742	100

murium, cannot utilize as a carbon source only 73 species of 600 compounds (8). The results here suggest that *Alcaligenes* bacteria, which are also soil inhabiting, are versatile in decomposing substances. *Alcaligenes* species have been found to utilize an unnatural compound, 2,4-dichlorophenoxyacetic acid (5, 16). However, little has been reported on the biochemistry and genetics of phenanthrene degradation by *Alcaligenes* isolates. In this study, a strain of *A. faecalis* was isolated which could degrade this hydrocarbon through the protocatechuate pathway. Interestingly, its Phn$^+$ phenotype was eliminated after successive subcultures of the wild strain with *o*-phthalate, one of the degradative intermediates of phenanthrene. This phenomenon is very similar to the curing of the TOL plasmid in *Pseudomonas putida (arvilla)* mt-2 with benzoate (20). Several other curing agents gave nonreversible Phn$^-$ strains, suggesting that the Phn$^+$ phenotype of strain AFK2 is dependent on plasmid-encoded genes.

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